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(54) Title: PROCESS FOR DETECTION, QUANTIFICATION AND/OR IDENTIFICATION OF A PEPTIDE

(57) Abstract

The invention relates to a process for detection, quantification and/or identification of a peptide which comprises the steps of labelling a peptide library on beads with a radioactive or light emittant substance, mixing with photographic media, spreading out the obtained slurry on a dish, incubation, developing and isolation of single labelled beads.

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PROCESS FOR DETECTION, QUANTIFICATION AND/OR IDENTIFICATION OF A PEPTIDE

The invention relates to a process for detection, quantification and/or identification of a peptide which comprises the steps of labelling a peptide library on beads with a radioactive or light emitting substance, mixing with photographic media, spreading out the obtained slurry on a dish, incubation, developing and isolation of single labelled beads.

INTRODUCTION

Using a recently developed technique, it is possible to produce peptide libraries that theoretically contain all possible sequence variants of a specified number of amino acids.

There are different types of peptides libraries. One is based on the presentation of a recombinant peptide on the surface of a bacteriophage fused with a phage protein and another is a solid phase combinatorial peptide library.

When the latter approach is applied, peptides are linked to a solid matrix of small plastic beads. In each coupling cycle, the beads are first divided into twenty portions of equal size and transferred to vials containing only one of the twenty amino acids found in mammalian proteins or other amino acids. After coupling of the amino acids to the beads, the reaction is halted and the beads from the twenty vials are mixed together in a single flask. They are then again split into twenty portions of equal size and the above procedure is repeated. At the end of a number of such cycles, the beads will all contain peptides of the same length, but of random sequences. Most importantly, all peptides on a

single bead will have the same sequence. Within a library of this type, some beads will contain peptides with specific biochemical, pharmacological and biological properties. However, the major obstacle is to identify single beads having a peptide with defined properties among tens of millions of nonrelevant beads.

In living cells, a large fraction of all proteins are modified by enzymes that covalently couple phosphate, sulphate, sugars, lipids, or other substances to one or several amino acid residues. These different chemical entities can all be made radioactive, for example by including one or several radioactive isotopes when they are synthesized. If such a radioactive substrate is used in the enzymatic reaction, the covalently modified protein will also become radioactive. In the search for unknown acceptor molecules, a library with peptides attached to plastic beads are much more convenient to use than a mixture of free peptides in solution.

Only beads containing peptides with physicochemical properties allowing them to interact correctly with the enzyme will become labelled. However, due to sterical hindrance and other obstacles, only a small fraction of the peptides on a bead can be modified in the enzymatic reaction. To identify a bead with a total radioactivity level of perhaps only a few disintegrations per minute among millions of nonradioactive beads, an extremely sensitive and specific method is required. We have solved this issue by developing a method for autoradiographic detection of single beads being radioactive or light emitting substance.

Kassarjian A, Peptide Research, Vol 6, No 3, 1993, p 129-132 discloses a method for screening of synthetic peptide libraries with radiolabelled acceptor molecules. Peptide resin beads, each carrying one peptide sequence, are incubated with radiolabelled acceptor molecule and thereafter poured on a thin layer of agarose. After incubation, a X-ray film was placed directly onto the

agarose film. The areas containing radioactive beads were cut out using a micropipe tip and combined. After a second step single positive beads were isolated.

This method does not allow a resolution which is sensitive to each single bead and is only indicating the area where the identified peptide can be found until a second step has been performed.

Our claimed method avoids the use of a X-ray film and the use of a two step procedure.

It also makes it possible to screen large libraries, up to 100,000,000 beads or more while Kassarjian works with 1,800,000 beads.

The claimed method is very selective. 1 out of 100,000,000 beads are visible.

The obtained beads are rather sticky,which makes the manual isolation of the beads very fast.

Figure 1 is illustrating a consensus sequence phosphorylated with radioactive ATP and the same peptide non-phosphorylated (Example)

Figure 2A is illustrating a radioactively labelled bead and non-labelled beads.

Figure 2B is illustrating the labelled and non-labelled beads in a higher magnification.

THE CLAIMED INVENTION

We claim a process for detection, quantification and/or identification of a peptide comprising the steps of

a) labelling a peptide library on beads with a radioactive or light emitting substance

b) mixing with a photographic media

c) spreading out the obtained slurry on a dish

d) exposure

e) developing and fixation and

f) isolation of single labelled beads

Step a) can comprise the following steps.

i) incubation of a peptide library on beads with selected enzyme and

ii) adding of radioactive or light emitting substrate

By substance is here meant an enzyme, ligand, antibody or other molecule capable of modifying or binding to the peptide.

The labelled beads surrounded by a halo of silver grains can be isolated, preferably crushed, and optionally thereafter amino acid sequenced.

The photographic media can be an emulsion.

The claimed process can be used for characterisation of recognition sequence for nonenzymatic covalent modifications of peptides, for characterisation of recognition sequence for enzymatic covalent modifications of peptides, for identification of receptors or binding proteins, for identification of peptide sequences capable of interacting with carbohydrates, for identification of molecules capable of modifying protein through removal or addition of amino acid residues from/to peptides, for identification of peptides sequences that may interact with intracellular signal transducers and for identification of peptides with catalytic properties.

EXPERIMENTAL PART

Materials and methods

Synthesis of peptide library

The combinatorial library was synthesized on TentaGel S RAM (polystyrene beads from Rapp Polymer; capacity 0.23 mmol/g) with a derivatized lysine providing the reactive amino group. For the generation of the library, we used the "split-synthesis" technique. Briefly, the peptides were synthesized using standard Fmoc methodology (See E & Sheppard RC, Solid phase peptide synthesis: a practical approach. Eds Atherton, IRL Press. Oxford, New York, Tokyo) in dimethylformamide (DMF) with 0.7 mmol of amino acids per gram of resin. Coupling was done in diisopropanolcarbodiimide, hydroxybenzotriazole,

and deblocking in 20% piperidine in DMF. Cysteine was omitted from the synthesis to eliminate cross-linking. We used the synthesis approach described by Lam et al. (1991) and Houghten et al. (1991). First, the beads were distributed into nineteen separate reaction vessels. A single amino acid species was then added to each reaction vessel. After completion of the coupling reaction (estimated by Kaiser's ninhydrin test), all beads were combined, mixed and deprotected. Thereafter, the beads were subdivided into nineteen new aliquots and the next residue was coupled. Totally, about 160 pmol of peptide was added to each bead.

Phosphorylation of peptides

The beads with the attached peptides were incubated in phosphorylation buffer (50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 15 mM 2-mercaptoethanol) containing 100 µM ATP and 1 µCi/ml of ^{32}P-gamma-ATP. The bead concentration was approximately 1 million beads per ml. The reaction was started by adding 50 units/ml of the catalytic subunit of cAMP-dependent protein kinase (Sigma Chemical Co.) and the reaction mixture was incubated at 30°C for 30 min. The reaction was stopped by addition of acetic acid in water to a final concentration of 10%. The beads were pelleted at 1 g, the supernatant removed, and "high salt buffer" added (phosphorylation buffer containing 1 M NaCl). The "high salt buffer" is necessary to remove ATP that otherwise binds nonspecifically to the beads.

However, the high salt wash causes the beads to form large clumps that make analysis of incorporation of ^{32}P into the peptides impossible.

Therefore, the beads are washed again in phosphorylation buffer containing 0.05% of the detergent Tween 20 to dissociate the clumps.

Autoradiography

The bead/detergent slurry is mixed with one to two volumes of photographic emulsion (Kodak NTB2, melted at 40°C) and poured out in a thin layer in glass or plastic dishes. Following air-drying, the dishes are placed in metal boxes and kept in the dark at 4°C to expose the photosensitive component in the emulsion to the emission from the radioactively labelled beads (the time required in this step has to be determined each occasion a new enzyme or substrate is used). At the end of this exposure, the dishes are allowed to adjust to room temperature (20°C), developed (Kodak D-19), fixed (Kodak Unifix), and finally rinsed in water. After air-drying, they are then ready for inspection. At this stage, a labelled bead will be found surrounded by a halo of silver grains and easy to detect even with the naked eye.

Identification of labelled beads

Beads surrounded by a halo of silver grains were isolated under a stereo microscope, using a pair of fine tweezers. They were then transferred to a sequencing cartridge. The N-terminal sequence was determined by adsorptive biphasic column technology, using an HP G1005A protein sequenator (Hewlett-Packard protein chemistry system, Palo Alto, CA, USA).

Example

Results

The catalytic subunit of cAMP-dependent protein kinase (cAPK) was used as enzyme to test if a peptide immobilized via its carboxy terminus can work as a substrate in this type of reactions. In an initial experiment, a well characterized heptapeptide substrate for cAMK called Kemptide (LRRASLG) was synthesized on a cellulose filter using the SPOT™technique (See Frank R, Tetrahedron (1992), 48:9217-32). This peptide was readily phosphorylated using

the incubation procedure described above . On Figure 1A can be seen the consensus sequence recognized by cAMP dependent protein kinase which was synthesised on a solid matrix and phosphorylated with radioactive ATP in situ.. In a parallell experiment, a Kemptide analogue lacking the phosphate acceptor serine (LRRAALG) was incubated under the same conditions. In this case very little, or no, ^{32}P was incorporated. Figure 1, right.

These data were interpreted to indicate that a peptide covalently bound to a matrix still retains its ability to serve as a protein kinase substrate.

Next, a combinatorial pentapeptide library was synthesized as described under Materials and methods.

It was incubated in the kinase reaction mixture and after 30 min the reaction was stopped by addition of acetic acid. The efficiency of each washing step was monitored by Cerenkov counting of the beads. Initially, the beads contained large quantities of radioactive ATP, that could be removed by repeated washing in "high salt buffer". This caused the beads to bind tightly to each other and form large clumps. These could be dissociated by washing the beads in buffer containing a low concentration (0.05%) of the non-ionic detergent Tween-20. Following the last wash (i.e. when the amount of radioactivity present in the beads was not possible to reduce further), the beads were resuspended in a small volume of water with 0.05% Tween-20 at a concentration of about 30% (v/v). Following mixture with photographic emulsion and air-drying, the emulsion was exposed for various time periods and then developed as described above. Here, exposure time varied between 12 and 72 h.

Fig. 2A shows a radioactively labelled bead surrounded by a halo of silver grains. Non-labelled beads are also visible in Figure 2A. At higher magnification, it is evident that the intensity is more pronounced in the immediate vicinity of the beads and weaker at a longer distance (Figure 2B). Following removal of the beads from the dishes, they were usually covered by a

thin layer of emulsion that was intensely stained by silver grains, hence giving a greyish or black colour to the bead.

The labelled beads were then isolated with a pair of fine tweezers and then crushed immediately before the transfer to the sequencing cartridge . Using this approach, we managed to obtain good recoveries in the sequencing procedure, usually ranging between 50 and 120 pmoles of amino acids per sequencing cycle.

DISCUSSION

We have thus demonstrated that peptides in a combinatorial peptide library that have been labelled with a radioactive isotope can be easily identified and isolated. In comparison with previously published methods, where the beads were immobilized in agarose and then subjected to autoradiography on a conventional film, our method gives a very high resolution. The reason is that the beads are in direct contact with the photographic media. This makes "subcloning" of the beads unnecessary. Instead they can be isolated directly.

Due to the fact that the peptides on the beads come in direct contact with the photographic medium, weak beta-emitting isotopes like tritium are sufficient to label the substrates. Nevertheless, isotopes emitting alpha or gamma rays, and molecules emitting visible or nonvisible light, can also be used for this purpose. Furthermore, it should be stressed that the method is not restricted to the analysis of covalent peptide modifications.
It may just as well be applied to the study of less stable interactions between peptides and other molecules. Here, four examples of such applications are mentioned:

(i) the binding of an antibody to its antigen;

(ii) the binding of hormones and other specific ligands to their receptors or other binding proteins;

(iii) the binding of a drug to a recognition site within a protein; and

(iv) the development of affinity ligands (i.e. finding peptide ligands to nonreceptor proteins or other molecules).

REFERENCES

Kit S. Lam et al. Nature (1991) 354:82-84 "A new type of synthetic peptide library for identifying ligand-binding activity."

Richard A. Houghten et al. Nature (1991) 354:84-86 "Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery."

CLAIMS

1. Process for detection, quantification and/or identification of a peptide comprising the steps of

a) labelling a peptide library on beads with a radioactive or light emitting substance

b) mixing with photographic media

c) spreading out the obtained slurry on a dish

d) exposure

e) developing and fixation

f) isolation of single labelled beads

2. Process according to claim 1 in which the substance is an enzyme, ligand, antibody or other molecule capable of modifying or binding to the peptide.

3. Process according to claim 1 or 2 in which step a) comprises

i) incubation of a peptide library on beads with selected enzyme and

ii) adding of radioactive or light emitting substrate

4. Process according to claim 1 in which the labelled beads surrounded by a halo of silver grains were isolated.

5. Process according to any of claims 4 in which the labelled beads surrounded by a halo of silver grains are isolated and thereafter amino acid sequenced.

6. Process according to claim 5 in which the labelled beads surrounded by a halo of silver grains are isolated, crushed and thereafter amino acid sequenced.

7. Process according to claim 1, in which the photographic media is an emulsion.

8. Process according to any of claims 1-7, in which the method is used for characterisation of recognition sequences for nonenzymatic covalent modifications of peptides.

9. Process according to any of claims 1-7, in which the method is used for characterisation of recognition sequences for enzymatic covalent modifications of peptides.

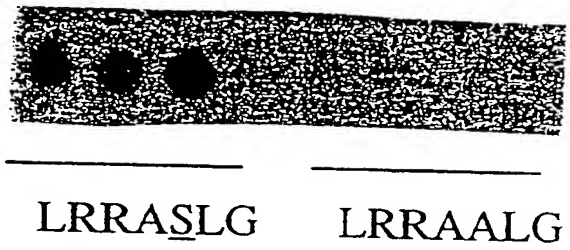
10. Process according to any of claim 1-7, in which the method is used for identification of receptors or binding proteins.

11. Process according to any of claim 1-7, in which the method is used for identification of peptide sequences capable of interacting with carbohydrates.

12. Process according to any of claim 1-7, in which the method is used for identification of molecules capable of modifying proteins through removal or addition of amino acid residues from/to peptides.

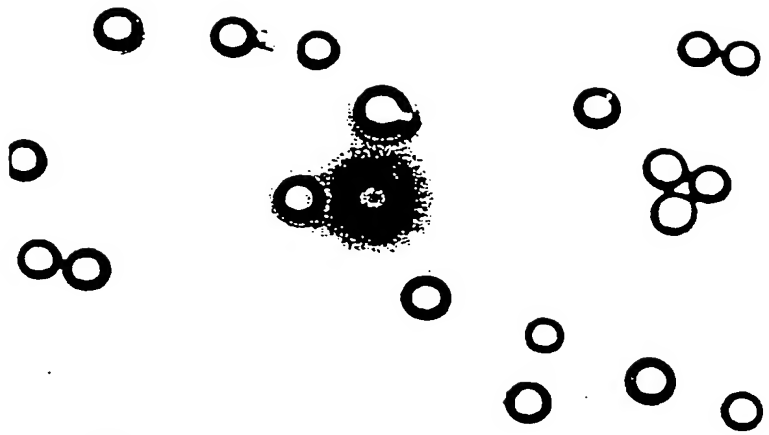
13. Process according to any of claim 1-7, in which the method is used for identification of peptides sequences that may interact with intracellular signal transducers.

14. Process according to any of claim 1-7, in which the method is used for identification of peptides with catalytic properties.

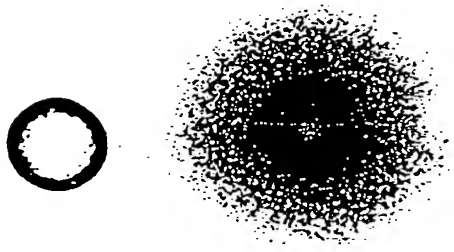


LRRASLG LRRAALG

Fig. 1



2A



2B

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/00780

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/543, G01N 33/58, C07K 1/04, G03C 1/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, USFULPAT, MEDLINE, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biopolymers (Peptide Science), Volume 37, 1995, Michal Lebl et al, "One-Bead-One-Structure Combinatorial Libraries", page 177 - page 198, see pages 180-181 --	1-13
A	WO 9200091 A1 (BIOLIGAND, INC.), 9 January 1992 (09.01.92) --	1-13
A	Biochemistry, Volume 33, 1994, Jinzi Wu et al, "Identifying Substrate Motifs of Protein Kinases by a Random Library Approach", pages 14825 - 14833; see paragraph briding pages 14826-27 -- --------	1-3,9,12

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9200091	09/01/92	AU-B-	659091	11/05/95
		AU-A-	2836995	07/12/95
		AU-A-	2845495	16/05/96
		AU-A-	8238591	23/01/92
		CA-A-	2086672	03/01/92
		EP-A-	0542770	26/05/93
		JP-T-	6500308	13/01/94
		NZ-A-	238805	26/07/94
		PL-B-	168354	29/02/96
		SK-A-	407392	10/08/94
		US-A-	5382513	17/01/95
		US-A-	5510240	23/04/96

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